AD_____

GRANT NUMBER DAMD17-96-1-6086

TITLE: A Novel Role for C/EBP in Mammary Development and Breast Cancer

PRINCIPAL INVESTIGATION: Cynthia A. Zahnow, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
A Novel Role for C/EBP in Mammary Development and Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6086

6. AUTHOR(S)
Cynthia A. Zahnow, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The objective of this fellowship is to characterize the roles which C/EBPα and C/EBPβ play in normal mammary gland development and breast cancer. In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells. LIP can act as a dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to excessive cellular proliferation. Our transplant studies of C/EBPα knockout tissue has demonstrated that C/EBPα is not necessary for normal mammary development; however, overexpression of C/EBPβ–LIP in the mammary gland of pregnant and lactating transgenic mice results in an abnormal mammary phenotype. Additionally, our research has confirmed that the C/EBPβ protein isoforms, and in particular, the LIP isoform, have been detected and are more highly expressed in aggressive, poorly differentiated infiltrating ductal carcinomas than in the less aggressive, better differentiated breast tumors. This observation is consistent with the hypothesis that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer C/EBPα, C/EBPβ-LIP, Dominant-Negative Transcription Factor, Cell Cycle, Transgenic Mice, Mammary Gland Development	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

CAZ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

CAZ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

CAZ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Cynthia C. Johnson 10/21/97
PI Signature Date

TABLE OF CONTENTS

INTRODUCTION

The failure of a cell to maintain balance between proliferation and terminal differentiation often results in tumor formation. The C/EBP family of DNA binding proteins plays a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation. In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells. LIP can act as a dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/LAP. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to excessive cellular proliferation. Consistent with this hypothesis, we have observed elevated LIP levels in several different mouse mammary tumors. These preliminary data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein, has prompted us to investigate the role of C/EBP in mammary gland tumorigenesis. We plan to determine whether the overexpression of LIP in both mammary cell lines and in transgenic mice can induce mammary tumorigenesis. Additionally, we will investigate the mechanisms by which hormonal factors are involved in this overexpression and how elevated levels of LIP may influence the transactivation potential of other C/EBP family members. We hypothesize that overexpression of LIP in mice may block terminal differentiation, and help facilitate uncontrolled proliferation and tumorigenesis. Finally, we will employ a novel combination of gene knockout and mammary gland transplantation technology to study the role of C/EBPα in regulating terminal differentiation.

The following specific tasks were proposed for the 36 months of this proposal. Due to the availability of mice, reagents and/or experimental difficulty, a few tasks were completed earlier than expected and some will take additional time.

Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis. Months 1-24. This requires 150 mice.

Task 1: Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP. (Months 1-6).

Task 2: Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice. (Months 6-12).

Task 3: Construction of the WAP-LIP-WAP construct. (Months 1-6).

Task 4: Generation and screening of transgenic mice which overexpress LIP. (Months 6-18).

Task 5: Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice. (Months 12-36).

Technical Objective 2: Determination of the effects of lactogenic hormones on C/EBPβ isoform expression, post-translational processing and functional activity in a mouse mammary epithelial cell line (HC 11). Months 1-12.

Task 6: Generation of a stably transfected mouse mammary cell line (HC 11) with a multimeric C/EBP promoter - CAT construct and treatment with lactogenic hormones. (Months 1-6).

Task 7: Analysis of C/EBPβ expression and activity in response to hormonal treatment. (Months 1-6).

Task 8: Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing. (Months 6-18).

Task 9: Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio. (Months 18-24).

Technical Objective 3: Analysis of the roles of C/EBP proteins on mammary gland development in a C/EBPα knockout mouse. Months 12-36. This requires 48 mice.

Task 10: Removal and transplantation of mammary glands from recipient C/EBPα mice into host 129 mice. (Months 12-24).

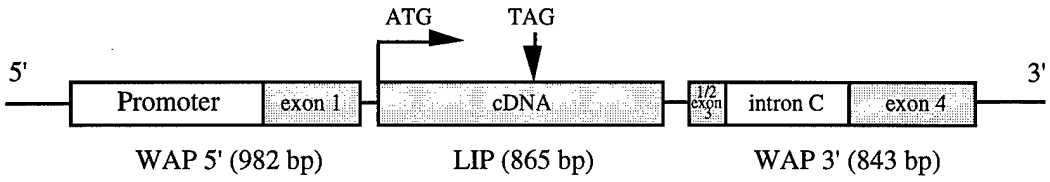
Task 11: Analysis of the development of mammary glands from C/EBPα knockout mice. (Months 24-36).

BODY

Experimental Methods

Construction of WAP-LIP-WAP Construct

The WAP promoter preferentially targets high levels of transgene expression to the mammary gland in transgenic mice, beginning at about day 10 of pregnancy and remaining elevated throughout lactation. Sequences in both the WAP promoter and the WAP 3' untranslated sequence have been demonstrated to be important for copy-number dependent transgene expression (3, 7). Consequently a WAP-LIP-WAP construct was created containing these sequences as follows: The first step in the generation of this construct was the *Eco R*I linearization and blunt ending of a pBluescript SKII(+) plasmid containing 843 bp of rat WAP 3' sequence containing a portion of the third exon, the third intron, all of the fourth exon and 70 bp of 3' flanking DNA. The second step included the removal of approximately 1 kb of an *Nco* I/*Xho* I LIP cDNA fragment from the C-terminal region of a mouse C/EBPβ (LAP) cDNA. The LIP cDNA contains the translation initiation Met codon and encodes a full length protein. Following blunt ending with Klenow, the LIP fragment was ligated to a position immediately 5' to the 3' WAP sequence in pSCPT SKII(+). In the third step, the LIP-WAP 3' construct was excised using both *Kpn* 1 and *Spe* 1, blunt ended and *Xba* 1 linkers were attached. This LIP-WAP3' fragment was then ligated to an *Xba* 1 linearized WAP 5' fragment, which consists of 982 bp of a rat WAP 5' promoter fragment (-949 to +1) and WAP 5' untranslated region (from +1 to +33). The integrity of the WAP-LIP-WAP construct was confirmed by sequencing the WAP-LIP boundaries. The WAP-LIP-WAP construct was removed from pSCPT SKII(+) by digestion with BstX I and Kpn I producing a vector (2.9 kb) and insert fragment (2.75 kb) which were similar in size. Further digestion using Pvu I, which only cuts the vector, allowed complete size fractionation and separation using agarose gel electrophoresis. The DNA was further purified and concentrated on a silica matrix (Glassmilk, Geneclean). Transgenic mice (FVB inbred) were generated by the transgenic core facility at Baylor College of Medicine.



Extraction of DNA from Mouse Tails

Tail tissue from either 1 day old or 3 week old anesthetized mice was digested at 55°C overnight in the following solution: (10mM Tris, pH 8.0, 75 mM NaCl, 25 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K). Genomic DNA was then extracted using a 1:1 mixture of STE-saturated phenol (150 mM NaCl, 100 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0) and chloroform:isoamyl alcohol (24:1). The DNA in the aqueous layer was ethanol precipitated, spooled out and resuspended in 1X TE (10mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

Polymerase Chain Reaction (PCR) Analysis of Tail DNA

Due to secondary structure in the template DNA, all PCR reactions were hot started in hot start tubes (Molecular Bio-Products). Each PCR reaction (25 µl) contained a bottom and top mix initially separated by a wax barrier. The bottom mix containing [1 µg genomic tail DNA, 1mM MgCl, 0.2mM dNTP's, 10% DMSO, and 10X Promega thermocycle buffer in a final volume of 14.5 µl] was heated to 90°C for 10 min, to denature the DNA and melt the wax pellet, and then cooled to 4°C. The top mix containing [12.5 pmoles of each primer, 10X Promega thermocycle buffer, and 2.5 U Taq polymerase (Promega) in a final volume of 10.5 µl] was added to the top of the hardened wax barrier and allowed to mix with the bottom reagents by heating to 94°C. The reaction profile was 30 cycles of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C. After the final cycle the samples were incubated at 72°C for an additional 5 min. Reactions were performed in a DNA thermocycler (Perkin Elmer-Cetus). The PCR products were resolved on a 1.5% agarose gel. The sequences of the synthetic oligonucleotides used in the PCR reactions were as follows (5' to 3'):rWAP+1(F), ATCAGTCATCACTTGCCTGCCGCCG and LIP 1574 (R), GTGTGTTGCGTCAGTCCCGTGTCCA.

Protein Extraction and Western Blot Analysis

Tissue and/or cells were disrupted in RIPA buffer (50mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% desoxycholate, 150 mM NaCl, 1 mM EGTA, 0.2% SDS) containing the following kinase, phosphatase and protease inhibitors; 1 mM $NaVO_3$, 1 mM NaF, 1 mM Na_2MoO_4, 10 nM okadaic acid, and 1µg/ml benzamidine, aprotinin, soybean trypsin inhibitor and antipain. Aliquots of these lysates containing 100 µg of protein were electrophoresed on denaturing SDS 12%-polyacrylamide mini-gels, then transferred to PVDF membranes (Millipore, Bedford, MA) overnight at 75 mA. Blots were blocked 90 min in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Tween-20) containing 3% non-fat dry milk (Carnation, Glendale, CA) then incubated for 90 min in this solution containing antibodies (0.5 ng/ml) (Santa Cruz) prepared against C/EBPβ. Blot washes consisted of TBST (without milk) three times for 5-10 min each, with agitation. Blots were then incubated for 60 min in blocking solution containing 200 ng/ml biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, England) and washed. Lastly, blots were incubated for 30 min in blocking solution containing 40 ng/ml streptavidin-horseradish peroxidase (Oncogene Science, Uniondale, NY) and washed as before. Enhanced chemiluminesence (Hyperfilm, Amersham) and chemifluoresence reagents (Storm Fluoroimager from Molecular Dynamics) were used for visualization as per the manufacturer's instructions.

Immunohistochemistry

Tissues were fixed for 6 hrs in 10% neutral-buffered formalin (NBF), embedded, sectioned, deparaffinized through a graded series of xylenes and alcohols and then rehydrated in water and phosphate-buffered saline. Antigens were retrieved by boiling for 10 min in 2 M urea, and endoperoxidases were blocked in a methanol solution containing 3% H_2O_2 for 15 min at room temperature. After washing, tissue was incubated for 1 hr at room temperature with 10% normal goat serum (NGS) (Sigma), and 20% avidin blocking solution (Vector Laboratories, Inc.) in 1X phosphate-buffered saline and 0.1% tween 20 (PBST). Excess blocking solution was drained and polyclonal rabbit primary antibody for C/EBPβ (1:300, Santa Cruz Biochemicals, Inc.) containing 20% biotin blocking solution (Vector Laboratories, Inc.) in 10% normal goat serum (NGS) was applied overnight at 4°C. Biotin-conjugated goat anti-rabbit secondary antibody at 1:200 dilution in 10% NGS was incubated for 30 min at room temperature. Biotin-avidin binding and DAB detection were carried out according to the manufacturer's instructions (Vector Laboratories, Inc.). Control slides were treated identically except that 6 μg of C/EBPβ peptide (Santa Cruz Biochemicals, Inc.) were incubated with the primary antibody for 15-30 min at 0°C.

Detection of Cellular Proliferation

Bromodeoxyuridine (BrdU) incorporation was used to label cells which have entered S phase. Evaluation of the number of mammary epithelial cells with BrdU incorporation aided in the determination of the level of cellular proliferation. Mice were anesthetized with 0.5-1.0 ml of Avertin and mammary glands were surgically removed 2 hrs after injection of 5-bromo-2'deoxyuridine (BrdU, *s.c.*, 0.3 mg/10 g body weight). Tissue was fixed in 10% neutral buffered saline for 6 hrs, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized through a graded series of xylenes and alcohols and then rehydrated in water and phosphate-buffered saline. Non-specific binding of tissue was blocked in 3% bovine serum albumin in 1X PBST for 15 min at 37°C and then rinsed in PBS for 1 min at room temperature. AntiBrdU containing nuclease (Amersham) was applied and sections were incubated for 90 min at 37°C. After three 1 min washes in PBST the peroxidase anti-mouse IgG was applied and sections were incubated for 30 min at 37°C washed as before and visualized using DAB-nickel according to the manufacturer's instructions (Vector Laboratories, Inc.).

Detection of Apoptosis

Tissue was fixed in 10% neutral buffered saline for 6 hrs, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized as described above and incubated with proteinase K (1 μg/ml) for 10 min at 37°C. Sections were washed 3X in PBST and blocked in PBS containing 10% MeOH, 0.3% H_2O_2 for 20 min at room temperature, washed in PBS as before and incubated with an end labeling mixture containing terminal deoxynucleotidyltransferase (TdT) buffer (30 mM Tris, pH 7.2, 140 mM NaCacodylate and 1 mM cobalt chloride), 1 nmol biotinylated dUTP, and 20 U of TdT for 60 min at 37°C. Labeled sections were incubated with ABC reagent according to the manufacturer's instructions and then incubated with DAB-nickel according to the manufacturer's instructions (Vector Laboratories, Inc.).

Mammary Gland Transplantation

An incision was made in the cleared, inguinal mammary fat pad of syngeneic, 3 week old, host +/+ 129/SvEv mice. Approximately 4 mammary glands were removed from either newborn -/- C/EBPα knockout mice or 8 week old +/+ 129/SvEv donor mice and inserted into fat pad pockets of the host. The overlying incision was closed with wound clips. Six to eight weeks after transplantation, a mammary gland was biopsied and examined for ductal tree growth and end bud formation. If local growth had occurred, the mice were bred to determine whether lobuloalveolar development would occur during pregnancy or were used as donors for serial transplantation.

Mammary epithelium which grew out from the original transplanted newborn tissue, was then used to transplant into another set of 3 week old 129/SvEv hosts (G1). Transplanted glands (G2) were then removed at the developmental time points of virgin (8 week), mid-pregnant, 1 day lactating and 1 day lactating followed by 4 days of forced involution and examined via whole mount analysis and 5 μm hemotoxylin and eosin stained sections.

Transfection of TM3 cells

TM 3 cells were grown and maintained using HEPES buffered D-MEM/F-12 growth media containing: 2% adult bovine serum, 10 μg/ml insulin, L-glutamine, 5 ng/ml epidermal growth factor (EGF), and 5 μg/ml gentamycin sulfate. Because mammary epithelial cells are inherently difficult to transfect, the transfection procedure had to be optimized. Cells were transfected with three different transfection reagents, Lipofectamine (Gibco, BRL), the Perfect Transfection series of pfx lipids (Invitrogen) and Superfect (Qiagen). It was determined that Superfect yielded the least toxicity and best transfection efficiency. At 20 to 40% of confluence, cells were stably transfected with 10 μg of the tet activator plasmid pUHD172-1neo using Superfect (Qiagen). Stably transfected cells were cloned and maintained with 0.2 mg G418 per ml growth media. TM3 cells are density dependent and hence take a very long time to expand when grown at the low density of a single colony. A total of 31 clones were then cotransfected with 10 μg of the tetracycline responsive plasmid pUHD10-3-LIP and 1 μg of the hygromycin selection vector pTK-Hyg. Each clone will be treated with or without doxycycline and tested via Western blot analysis for the production of tetracycline regulatable LIP protein.

Results and Discussion

Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis. Months 1-24.

To test the relevance of the mouse model, we have extended technical objective 1 to include studies of human breast cancer.

To address the question, **"Is LIP expressed in human breast cancer?"**, infiltrating ductal carcinomas from 39 women, aged 26 to 83 years old were analyzed for C/EBPβ expression. As evidenced by Western blot analyses, LAP and LIP expression levels within the infiltrating ductal carcinomas were quite variable among the different patients (Fig. 1A, B and C). Additional protein bands, representing both cross-reactive proteins and phosphorylated or other post-translationally processed LAP and LIP isoforms were also visible in many tumors.

To determine the significance of elevated LIP expression in some tumors, an expression level was assigned to the LIP protein values and was tested for association with various prognostic factors. LIP was expressed at high levels in 23% (9/39) of the infiltrating ductal carcinomas. Examples of high LIP expression are shown in Fig. 1B lanes 2, 3 and 6. LIP levels designated as "high" ($1.59 \pm .6$) were on average 15 times greater than the levels of LIP in the lower ($0.107 \pm .02$) and non-expressing tumors ($P < .0001$, two-sided Mann-Whitney t-test). In "normal" breast tissue, LIP levels were low or not detectable above background values.

Because the amino-truncated LIP isoform has a greater DNA affinity than the LAP isoforms and can heterodimerize and inhibit the transactivation ability of other C/EBPs at a substoichiometric ratio as low as 1 : 5 (20%), the LIP/LAP ratio was determined for the predominant LAP isoform (35 kDa) which also has the greatest transactivation potential (4) . In the tumors expressing the highest LIP levels the average ratio of LIP/LAP was determined to be 1: 2.4 ($42.3\% \pm 6\%$).

Two of the most frequently used prognostic indicators in breast cancer are tumor size and lymph node status. No association was observed between these indicators and C/EBPβ-LIP levels. Unfortunately, the tissues analyzed from the Methodist Hospital tissue bank were biased towards tumors larger than 1 cm because of the inherent difficulties in banking tissue from smaller tumors. Consequently tissue was analyzed from tumors ranging in size from 1.2 cm to 20 cm at the largest dimension. Additionally, 22 patients had metastases to the lymph node and the nodal status for 11 patients was not determined. The sample size for the six node-negative patients and <1 cm tumors was, therefore, not large enough to draw any statistically significant conclusions.

To determine whether an association existed between genetic instability and LIP expression in the infiltrating ductal carcinomas, LIP expression levels in 38 of 39 tumor specimens were compared to DNA ploidy. Nine (100%) of the nine tumors which expressed LIP at high levels were aneuploid. The tumors which either expressed LIP at low or non-detectable levels were more evenly distributed with 17 (59%) of 29 aneuploid in contrast to 12 (41%) of 29 diploid ($P = .0356$, two-sided Fisher's Exact test).

Because loss of estrogen receptor (ER) expression is often associated with a poor clinical outcome (5), it was next determined whether steroid receptor status in these tumors was associated with LIP expression. Of the 39 infiltrating ductal carcinomas examined, 14 (36%), of 39 specimens were negative for estrogen and progesterone receptors (ER^-/PR^-), and 19 (49%) of 39 were positive for estrogen and progesterone receptors (ER^+/PR^+). Eight (89%) of nine ER^-/PR^- tumors expressed high levels of LIP, whereas only one (11%) of 9 ER^+/PR^+ tumors exhibited high levels of LIP expression. ($P = 0.0015$, two-sided Fisher's Exact test) (Table 1).

Each tumor was blindly graded by use of the Elston/Ellis system (6) which examines and scores the degree of tubule formation, the degree of nuclear pleomorphism, and the number of mitotic counts. All of the high LIP expressing tumors (9/9) were classified as poorly differentiated (i.e., Elston/Ellis grade III) and highly proliferative tumors. In contrast, all three histological grades (grade III - 36.7%, grade II - 30%, and grade I - 33.3%) were evenly distributed between the tumors expressing LIP at low or non-detectable levels ($P = .013$, two-sided Fisher's Exact test). The proliferative fraction, determined by either MIB-1 immunohistochemistry and/or by DNA flow cytometry, also displayed an association with LIP expression. In the infiltrating ductal carcinomas which contained high LIP levels, eight (89%) of 9 specimens contained a high fraction of proliferative cells, but only eight (30%) of 27 of the low and non-detectable LIP expressing tumors contained a high fraction of proliferative cells ($P = .0046$, two-sided Fisher's Exact test).

Although it was not possible to localize LIP expression to specific cell types, immunocytochemical staining was performed on a limited number of the infiltrating ductal carcinomas and surrounding tissue to determine which cells expressed the C/EBPβ isoforms. These results were consistent with the previous Western analyses and revealed strong C/EBPβ expression in pleomorphic nuclei of grade III, ER^-/PR^- tumors (Fig. 2A) and weaker C/EBPβ expression in the nuclei of grade I, ER^+/PR^+ tumors (Fig. 2B). Surrounding normal tissue also expressed C/EBPβ in both the epithelium and stroma (data not shown). The specificity of the staining was confirmed by selective competition with the peptide against which the carboxy-terminal antisera was generated, but not by a non-specific peptide (data not shown). These results from immunohistochemical analyses have confirmed that the C/EBPβ isoforms are strongly expressed in malignant epithelial cells of aggressive, poorly differentiated infiltrating ductal carcinomas (Fig. 2A, B). However, development of a double fluorescent imaging technique using amino- and carboxy-terminal antisera will be required to detect the selective localization of LIP expression in the future. Examination of hematoxylin and eosin stained microscopic tissue sections of both the high and low LIP-expressing tumors also demonstrated that many of the less aggressive, low LIP expressing tumors possessed large sheets of tumor epithelium as illustrated in Fig. 2. In previously published studies (9) using clonal mouse mammary epithelial cell lines, it

was demonstrated that differences in the LIP/LAP ratio cannot be accounted for by variations in epithelial cell number.

Task 1: Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP. (Months 1-6).

These experiments are difficult because although early passage TM 3 cells have low basal levels of LIP, later passage cells can become transformed and LIP expression may increase. Consequently, it is necessary to generate these cells in as few steps as possible. Clonal TM3 cells have been generated as described in Experimental Methods and we are currently expanding these clones so that we may test them for inducibility by doxycycline. If these clones are non-inducible and constitutively express LIP then we will generate additional stables with a retroviral tetracycline system vector. Retroviruses are desirable because they are more efficient at transfecting mammary epithelial cells and integrate at only one copy per cell. Additionally, only a single transfection with a construct containing both the tet activator and LIP cDNA is needed.

Task 2: Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice. (Months 6-12).

This task has not been initiated because we have not yet isolated a TM3 clone with tetracyline regulated LIP expression.

Task 3: Construction of the WAP-LIP-WAP construct. (Months 1-6).

This task has been completed (See Experimental Methods).

Task 4: Generation and screening of transgenic mice which overexpress LIP. (Months 6-18).

Transgenic mice (FVB inbred) were generated by the transgenic core facility at Baylor College of Medicine. Mice were screened for the presence of the LIP transgene by PCR analysis of genomic tail DNA (See Experimental Methods). Three lines of transgenic mice which overexpress the LIP protein in their mammary glands have been generated. Western blot analysis has determined that line (6067) is the highest expressing line and lines (6070) and (6074) are lower expressing. Several other lines contained the transgene, but did not express it. Investigation of the mammary gland phenotype can only occur in mice which are or have been pregnant because the WAP promoter which preferentially targets high levels of transgene expression to the mammary gland, drives expression at about day 10 of pregnancy and continues throughout lactation.

Task 5: Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice. (Months 12-36).

Although the analysis of the LIP phenotype is ongoing, some preliminary results have been obtained. Several questions were addressed in this task.

1. Does overexpression of LIP in the mammary gland interfere with lactation?

Observations of mice which are over one year in age and have had a maximum of 6 litters, have demonstrated that this protein has no affect on the ability of the mice to nurse their young. Litter sizes and pup weights appear to be normal. In addition, preliminary results obtained by Western blot analysis, have demonstrated that β-casein levels are unchanged in pregnant and lactating glands of transgenic mice. The mammary gland has a tremendous ability to compensate for changes in its environment. Perhaps the LIP expression level is not high enough to overcome the transactivation potential of all of the other C/EBP family members.

2. Does overexpression of LIP induce mammary tumors?

We have repeatedly bred several females in order to maintain the LIP expression level and facilitate tumor formation. However, after approximately 1 year of breeding no tumors have been observed.

3. Does overexpression of LIP alter normal mammary gland development?

Examination of hematoxylin/eosin stained 5μm tissue sections have demonstrated that mice which overexpress LIP in their mammary glands have an altered or abnormal phenotype. In 6 day lactating tissue, the alveoli appear to be smaller or more condensed than in control animals (Fig.3). (Control mice for all experiments consisted of wild type FVB mice and/or non-transgenic littermates). Additionally, luminal secretions appear to be reduced and abnormal. The shape of many of the alveolar cells is distorted and the cytoplasmic to nuclear ratio may be altered. Additionally, there is an increase in the stromal compartment. This may represent a decrease in the ability of the epithelial cells to completely fill out the mammary fat pad. This phenotype has been observed as early as day 18 of pregnancy and as late as day 10 of lactation.

4. Does overexpression of LIP affect cellular proliferation, terminal differentiation or apoptosis?

Immunohistochemical studies on paraffin embedded mammary samples are in progress.

5. Analysis of LIP overexpression on mammary gland development in transplanted BALB/c mice has not been initiated.

Tasks 6, 7, 8 and 9 are still in progress.

Task 6: Generation of a stably transfected mouse mammary cell line (HC 11) with a multimeric C/EBP promoter - CAT construct and treatment with lactogenic hormones. (Months 1-6).

Task 7: Analysis of C/EBPβ expression and activity in response to hormonal treatment. (Months 1-6).

Task 8: Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing. (Months 6-18).

Task 9: Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio. (Months 18-24).

Tasks 10 and 11. Removal and transplantation of mammary glands from recipient C/EBPα mice into host 129 mice and analysis of the development of mammary glands from C/EBPα knockout mice. (Due to the availability of knockout mice and technical expertise it was necessary to initiate these tasks in year 1 instead of years 2 and 3).

Targeted disruption of C/EBP gene expression can provide valuable information about the specific roles of each C/EBP family member in cellular differentiation and proliferation. Recently, both C/EBPβ and α knockout mice have been generated (11, 13, 15). The phenotype of the C/EBPβ deleted mouse consists of reproductive defects, (8, 12) an immunological disorder which results in defective macrohage activation, and lymphoproliferative / myeloproliferative alterations (11, 13). The phenotype of the C/EBPα knockout has demonstrated that the C/EBPα protein regulates metabolic fuel levels in the neonate and is critical for glycogen and lipid storage (15). Consequently, homozygous, C/EBPα deleted, neonatal mice become hypoglycemic at 1 to 2 hours after birth and die by 8 hours postpartum (15). If the mice are subcutaneously injected with 10% glucose every 7 hours, they can survive for up to 40 hours. This poor survival rate renders it impossible to study mammary gland development in the absence of C/EBPα expression in these

homozygous mutant mice. Consequently, it was necessary to remove and transplant mammary glands from recipient C/EBPα mice into host 129 mice in order to study the role of C/EBPα in mammary gland development.

Several questions were addressed in these tasks.

1. Is C/EBPα necessary for mammary gland growth and differentiation?
No, it does not appear to be required because the glands exhibited normal growth and morphology at all developmental time points examined. No obvious defects were observed when comparing the -/- transplants to +/+ transplants or when comparing the -/- transplants to the #3 glands (internal control) of the hosts.

2. Is C/EBPα necessary for milk production?
No, it is not necessary because secretions were observed in both the +/+ and -/- transplanted glands of 1 day lactating mice. Additionally, Western blot analysis did not detect a difference in the levels of β-casein, an abundant milk protein, in tissue extracts from 1 day lactating +/+ and -/- mice. Finally, hematoxylin and eosin stained sections of paraffin embedded 1 day lactating tissue demonstrated proteinaceous and lipid-like secretions inside the lumen of alveoli in both the +/+ and -/- transplants. Therefore, secretion does not appear to be affected in the transplants.

3. Is C/EBPα necessary for the apoptosis which naturally occurs during mammary gland involution?
We hypothesized that if cells must be terminally differentiated in order to undergo apoptosis and C/EBPα is important in differentiation, then apoptosis rates might decline in the C/EBPα -/- mice. Using TUNEL analysis on paraffin embedded, 5 μm sections, from transplants of 4 day involuted glands, we did not observe any difference between the number of apoptotic cells in the -/- mice vs. +/+ mice. The mammary glands from both groups of mice contained about 2.5% apoptotic cells / total number of cells.

In conclusion, our research has demonstrated that C/EBPα is not required for normal mammary gland development, or milk production in lactating mice. However, other C/EBP family members, such as C/EBPβ, may be able to compensate for the lack of actions by C/EBPα. The question of why C/EBPα protein is expressed almost exclusively during lactation remains to be answered.

CONCLUSIONS

Our research has confirmed that the C/EBPβ protein isoforms, and in particular, the naturally-occurring dominant-negative LIP isoform, have been detected and are more highly expressed in aggressive, poorly differentiated infiltrating ductal carcinomas than in the less aggressive tumors. This observation is consistent with the hypothesis that the C/EBPβ-isoforms may play a role in regulating terminal differentiation and cell cycle progression as illustrated in Fig. 4. Proliferative diseases such as cancer, are often the result of failure to withdraw from the cell cycle at the G_1 checkpoint. Multiple signal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late G_1 is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). Recent studies (2) have demonstrated that Rb directly interacts with and activates all of the C/EBPβ isoforms; however, it is not known how this interaction affects Rb activity. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland, and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. We, therefore, propose that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression

(Fig. 4). Support for this hypothesis comes from the observations that the LIP/LAP ratio is regulated during proliferative phases of both liver and mammary gland development (1, 4, 10)

These studies do not answer the question of whether overexpression of LIP, in the most advanced, aggressive tumors, facilitates tumor progression or if overexpression is simply a result of increased proliferation. These type of functional studies cannot be performed with patient biopsies, but require the use of animal or cell culture models. In this regard, we have generated transgenic mice that selectively express high levels of LIP in cells of the mammary gland and are analyzing the altered mammary gland phenotype. Because tumorigenesis is often the sum of multiple genetic "hits" over an extended period of time, it may take several years for our mice to develop mammary gland tumors. Consequently, we are still monitoring our relatively young C/EBPβ-LIP mice for mammary gland tumors. Currently, we are breeding these mice with C/EBPβ knockout mice to study the function of LIP in a background of reduced C/EBPβ-LAP. We hypothesize that the phenotype observed in these mice may be more severe than that observed in just the LIP transgenics and we are also monitoring these mice for tumor formation. Additionally, we are also breeding the C/EBPβ–LIP mice with transgenic mice expressing mutated p53 (172^{R-H}) which has been targeted to the mammary gland with the WAP promoter. These mice show increased susceptibility to mammary carcinogenesis when induced by DMBA and the resulting tumors have increased genomic instability (Li *et al.*, in press). We hope that these bigenic mice will provide some insight of the role C/EBPβ-LIP plays in the cell cycle. Studies are also in progress to determine the effects of regulatable LIP expression on cell cycle progression in mammary epithelial (TM3) cells. These types of experiments will be required to determine if LIP expression is a cause or effect of mammary tumorigenesis. Notwithstanding this determination, LIP expresssion may provide a useful additional marker for the identification of breast tumors in patients with a poor clinical outcome.

REFERENCES

1. Buck, M., H. Turler, and M. Chojkier. 1994. LAP (NF-IL6), a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. EMBO J. 13:851-860.
2. Chen, P. L., D. J. Riley, S. Chen-Kiang, and W.-H. Lee. 1996. Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA 93:465-469.
3. Dale, T. C., M. J. Krnacik, C. Schmidhauser, C. L. Yang, M. J. Bissell, and J. M. Rosen. 1992. High-level expression of the rat whey acidic protein gene is mediated by elements in the promoter and 3' untranslated region. Mol Cell Biol 12:905-14.
4. Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67:569-579.
5. Elledge, R. M., G. M. Clark, G. C. Chamness, and C. K. Osborne. 1994. Tumor biologic factors and breast cancer prognosis among white, Hispanic, and black women in the United States. Journal National Cancer Institute 86:705-711.
6. Elston, C. W., and I. O. Ellis. 1991. Pathological prognostic factors in breast cancer. 1. The value of histological grade in breast cancer: experience from a large study with long-term follow up. Histopathology 19:403-410.
7. Krnacik, M. J., S. Li, J. Liao, and J. M. Rosen. 1995. Position-independent expression of whey acidic protein transgenes. J. Biol. Chem. 270:11119-11129.
8. Pall, M., P. Hellberg, M. Brannstrom, M. Mikuni, C. M. Peterson, K. Sundfeldt, B. Norden, L. Hedin, and S. Enerback. 1997. The transcription factor C/EBP-β and its role in ovarian function; evidence for direct involvement in the ovulatory process. EMBO 16:5273-5279.

9. Raught, B., A.-C. Gingras, A. James, D. Medina, N. Sonenberg, and R. J.M. 1996. Expression of a translationally regulated, dominant-negative CCAAT/enhancer-binding protein β isoform and up-regulation of the eukaryotic translation initiation factor 2a are correlated with neoplastic transformation of mammary epithelial cells. Cancer Res. 56:4382-4386.
10. Raught, B., W. S.-L. Liao, and J. M. Rosen. 1995. Developmentally- and hormonally-regulated C/EBP isoforms influence β-casein gene expression. Mol. Endo. 9:1223-1232.
11. Screpanti, I., L. Romani, P. Musiani, A. Modesti, E. Fattori, D. Lazzaro, C. Sellitto, S. Scarpa, D. Bellavia, G. Lattanzio, F. Bistoni, L. Frati, R. Cortese, A. Gulino, G. Ciliberto, F. Costantini, and V. Poli. 1995. Lymphoproliferative diosorder and imbalanced T-helper response in C/EBPβ -deficient mice. EMBO J. 14:1932-1941.
12. Sterneck, E., L. Tessarollo, and P. F. Johnson. 1997. An Essential Role for C/EBPβ in Female Reproduction. Genes and Development 11:2153-2162.
13. Tanaka, T., S. Akira, K. Yoshida, M. Umemoto, Y. Yoneda, N. Shirafuji, H. Fujiwara, S. Suematsu, N. Yoshida, and T. Kishimoto. 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell 80:353-361.
14. Umek, R. M., A. D. Friedman, and S. L. McKnight. 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. Science 251:288-292.
15. Wang, N.-d., M. J. Finegold, A. Bradley, C. N. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, and G. J. Darlington. 1995. Impaired energy homeostasis in C/EBPα knockout mice. Science 269:1108-1115.
16. Yeh, W.-C., Z. Cao, M. Classon, and S. L. McKnight. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 9:168-181.

FIGURE LEGENDS

Figure 1. Western blot analysis and ECL detection of C/EBPβ isoforms (LAP - 35kDa and LIP - 20kDa) in human infiltrating ductal carcinomas. Each lane represents the infiltrating ductal carcinoma from a different patient with the exception of lanes 1 and 2 in panel A. These lanes consist of an infiltrating ductal carcinoma and its paired, normal (N), tissue. One hundred μg of protein were analyzed per lane. The estrogen (ER) and progesterone receptor (PR) status from each infiltrating ductal carcinoma is listed above the corresponding lane. The exposure time for figure 1B is not the same as for 1A and 1C and these Western blots were not all performed simultaneously. The variations in LIP levels on these ECL blots have been confirmed using the quantitative ECF methodology described in the Methods.

Figure 2. Immunohistochemical localization of C/EBPβ isoform expression in a human (A) Elston/Ellis grade III, ER^-/PR^-, infiltrating ductal carcinoma and (B) an Elston/Ellis grade I, ER^+/PR^+, infiltrating ductal carcinoma. Note the intense staining of pleomorphic nuclei in (A) and much weaker staining in (B). In agreement with these results, western blot analysis of the infiltrating ductal carcinoma in panel (A) shows strong expression of the C/EBPβ isoforms (Fig. 1B, lane 6), but the infiltrating ductal carcinoma in (B) expresses the isoforms at a much lower level (Fig. 1A, lane 3). Viewed with a 40X objective.

Figure 3. Hematoxylin and eosin stained (5 μm) sections of mammary glands from day 6 of lactation. A. Mammary gland tissue from a non-transgenic littermate (high expressing line) showing large, open alveolar lumens and cuboidal epithelial cells lining the alveoli. Panels B,C,and D show mammary gland tissue from mice of the high expressing (B) and lower expressing (C and D) C/EBPβ-LIP lines. Notice the smaller alveolar lumens, increased amount of adipose tissue between the alveoli, and altered shape, size and organizational pattern of the epithelial cells.

Figure 4. A hypothetical model for the role of C/EBPs in cell cycle progression. The interaction of retinoblastoma protein with the C/EBPβ–LAP and LIP isoforms may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland (see text for details). Studies in adipocytes have demonstrated that C/EBPβ and C/EBPδ are involved in early proliferative and differentiative processes leading to the activation of C/EBPα which then contributes to terminal differentiation by arresting adipocyte proliferation (14, 16). In HepG2 hepatoma cells, C/EBPβ -LAP, has been reported to inhibit cell cycle progression before the G_1/S boundary and this effect can be antagonized by expression of the dominant-negative LIP isoform, thereby promoting cellular proliferation (1). In the rat mammary gland, C/EBPα expression is highest at lactation, when mammary epithelial cells undergo terminal differentiation, and C/EBPβ expression is highest during pregnancy, a period of lobuloalveolar proliferation (10) The LIP/LAP ratio decreases almost 100-fold at the onset of lactation.

Table 1. Association of LIP expression levels with steroid receptor status.

Receptor Status	LIP Expression Level	
	High	Low and N/D
ER^-/PR^-	89% (8/9)	20% (6/30)
ER^+/PR^+	11% (1/9)	60% (18/30)
ER^-/PR^+	NONE	7% (2/30)
ER^+/PR^-	NONE	13% (4/30)

Association of steroid receptor status with LIP overexpression in infiltrating ductal carcinomas. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) were negative for estrogen and progesterone receptor (ER^-/PR^-), 19 (49%) were positive for estrogen and progesterone receptor (ER^+/PR^+), and 6 (15%) were combinations thereof. (High) LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, (Low and non-detectable N/D) LIP values ranged from 0.3-fold below standard values to 0. Frequency analysis was performed using a 2-tailed Fisher's Exact test ($P = .0015$).

Figure 1

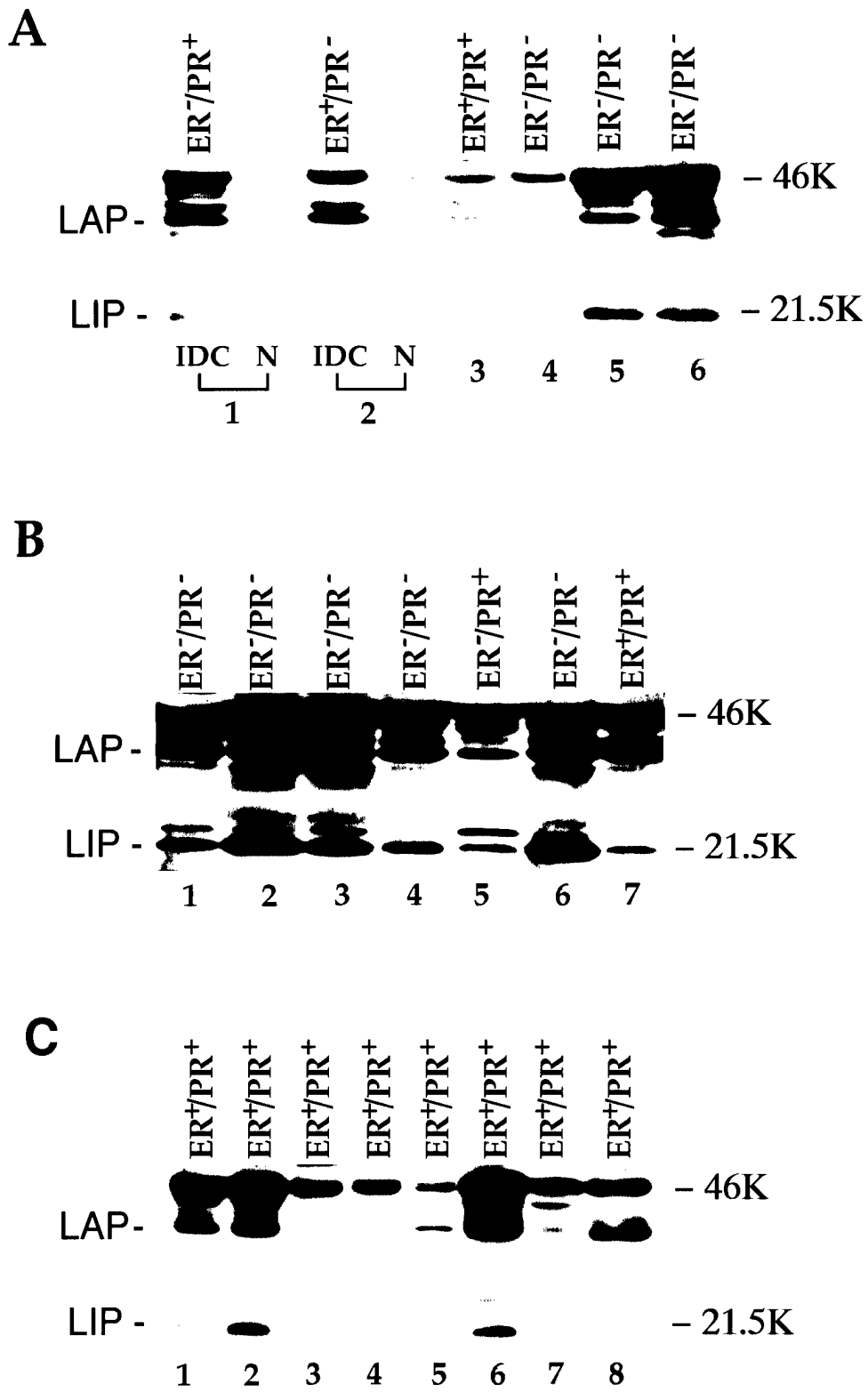

A
ER-/PR+
ER+/PR-
ER+/PR+
ER-/PR-
ER-/PR-
ER-/PR-
LAP-
– 46K
LIP -
– 21.5K
IDC N
IDC N
1
2
3
4
5
6
B
ER-/PR-
ER-/PR-
ER-/PR-
ER-/PR-
ER-/PR+
ER-/PR-
ER+/PR+
LAP-
– 46K
LIP -
– 21.5K
1
2
3
4
5
6
7
C
ER+/PR+
ER+/PR+
ER+/PR+
ER+/PR+
ER+/PR+
ER+/PR+
ER+/PR+
ER+/PR+
LAP-
– 46K
LIP -
– 21.5K
1
2
3
4
5
6
7
8

Figure 2

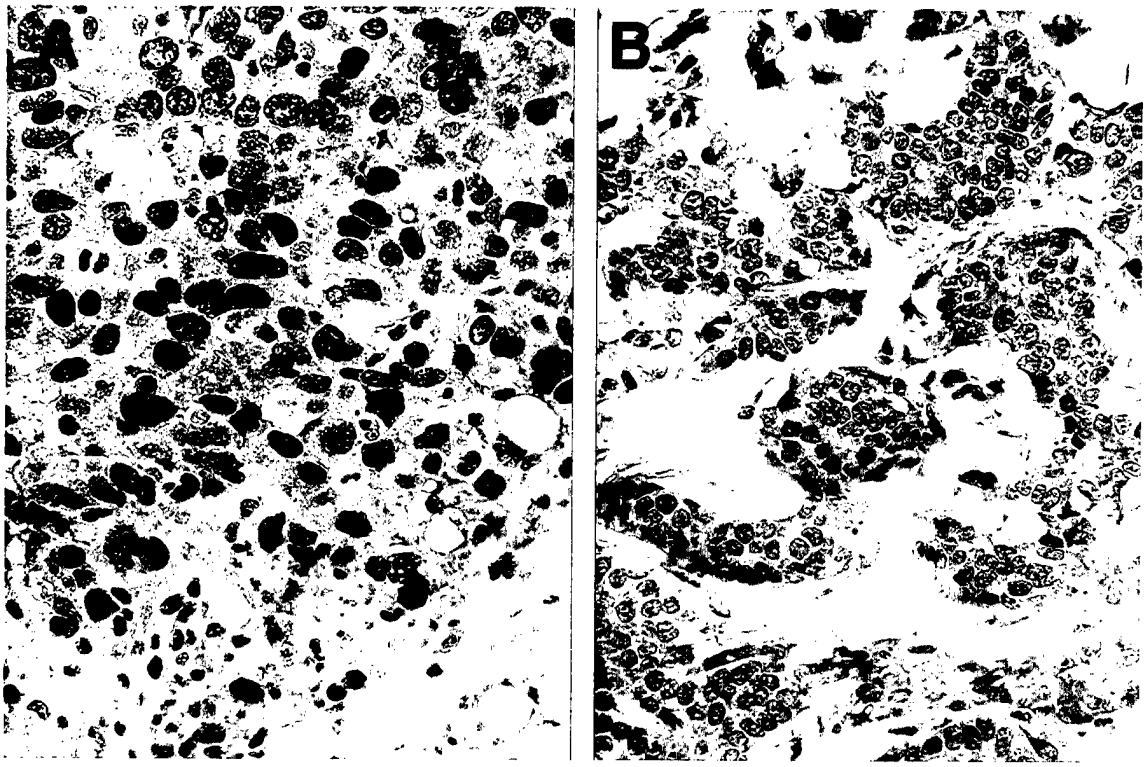


Figure 3

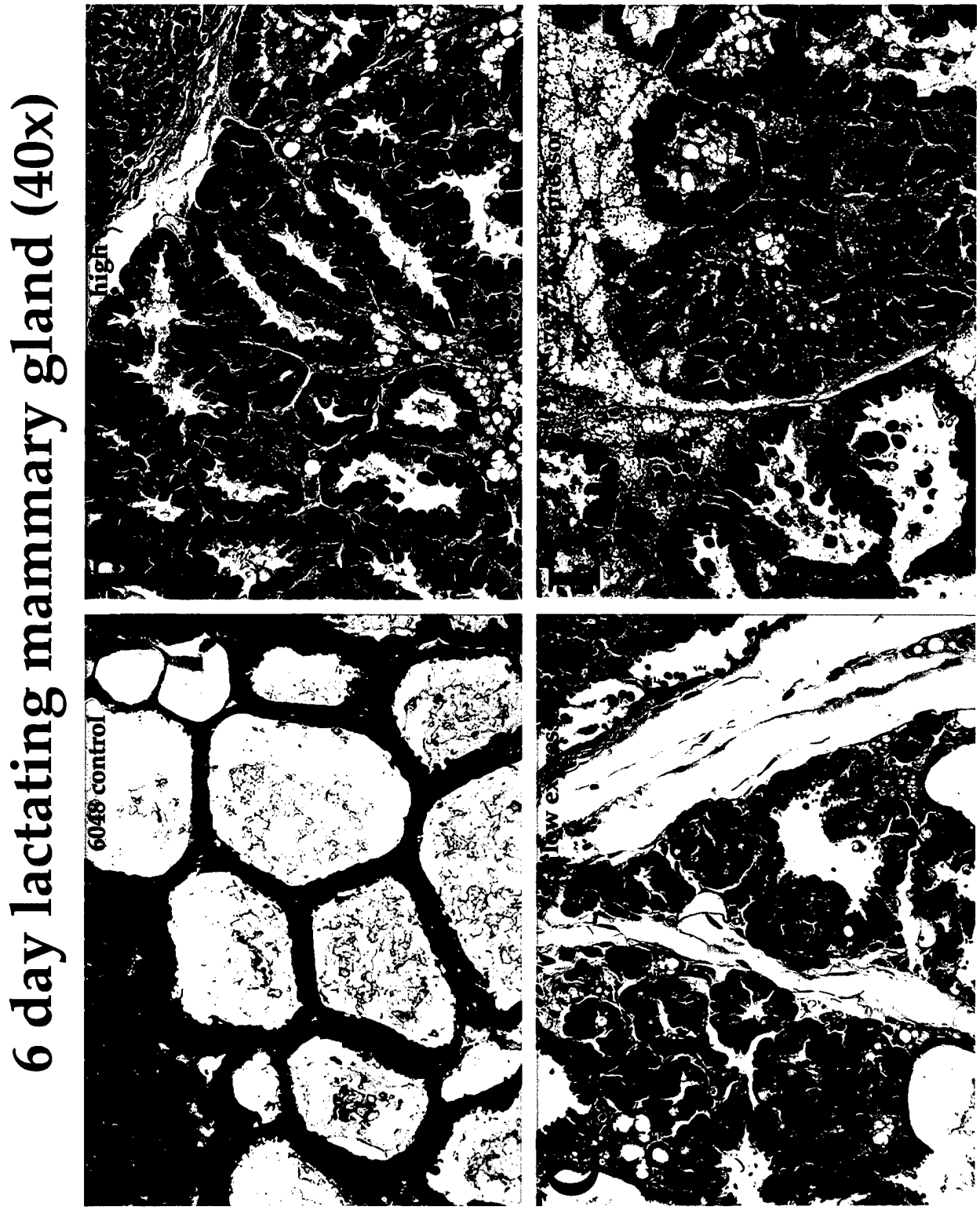
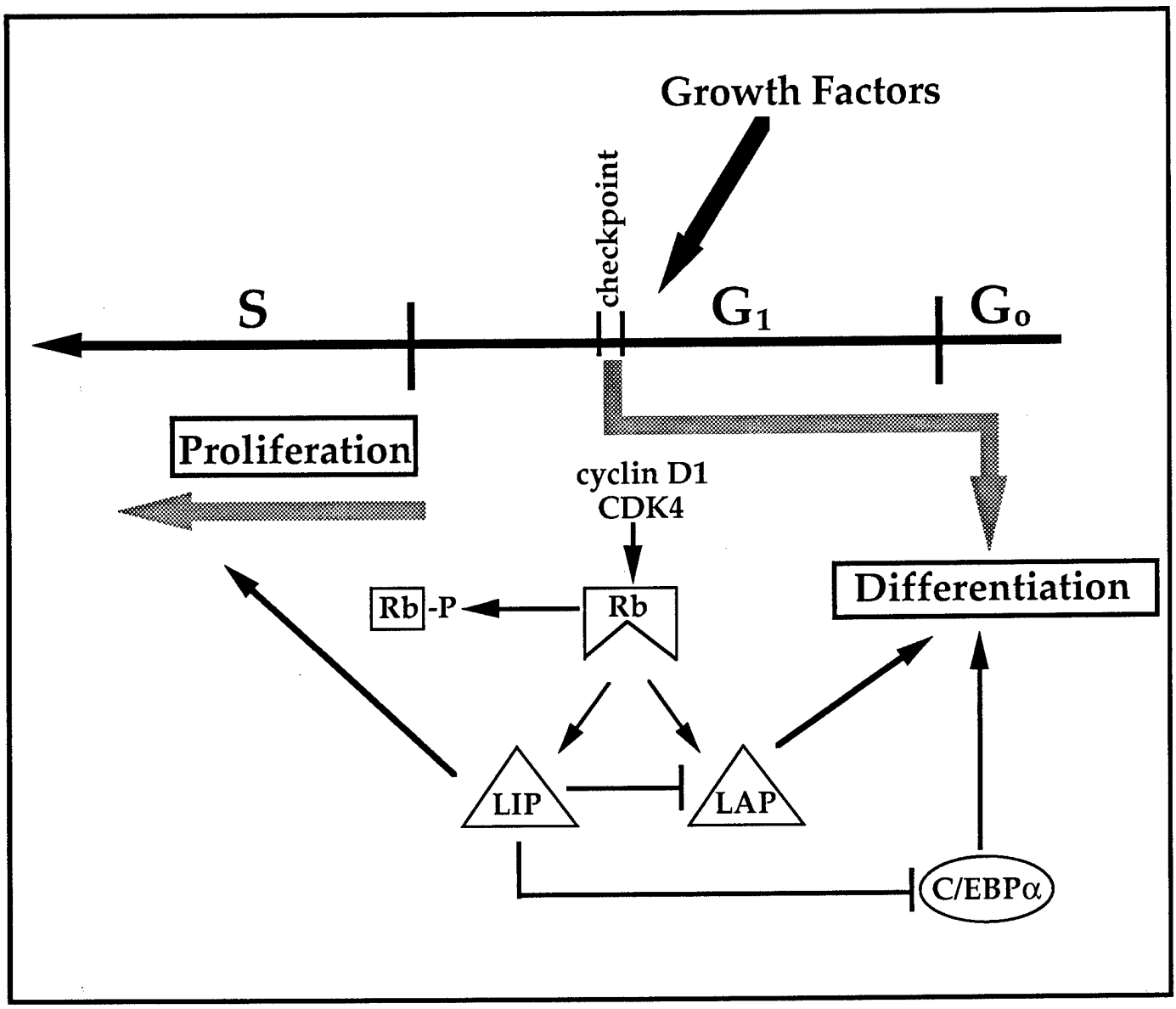


Figure 4


Growth Factors
checkpoint
S
G1
Go
Proliferation
cyclin D1
CDK4
Differentiation
Rb-P
Rb
LIP
LAP
C/EBPα